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Analysis of Hepatitis B Virus Genotypes and Pre-Core Region Variability During Interferon Treatment of HBe Antigen Negative Chronic Hepatitis B

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The clinical importance of hepatitis B virus (HBV) genome variability has been reported recently. One example is the occurrence of hepatitis B virus pre-core mutants, which arise during spontaneous or interferon-induced seroconversion from HBeAg to anti-HBe and are thought to be selected by immune pressure. A survey of HBV pre-core mutants and viral genotypes in 35 HBeAg negative patients during interferon therapy was carried out to understand viral pathogenesis in this form of chronic hepatitis B. Seventeen patients responded to interferon therapy as assessed by the sustained normalization of serum ALT levels and the significant decrease of viremia levels. The response rate to interferon was independent of both initial serum viral DNA level and interferon doses. During interferon therapy, a significant decrease of M0 (wild-type pre-core sequence at pos. 1887–1908), M1 (TGG to TAG at pos. 1896) or M2 (TGG to TAG at pos. 1896, and GGC to GAC at pos. 1899) positive viral genomes was found in 48%, 42%, and 33% of patients, respectively. A higher response rate to interferon therapy was observed in patients infected with HBV genotype A (70%) or M0 positive strains (75%) as compared to patients infected with genotype D/E (40%) or M1/M2 positive strains (44%). The data support the hypothesis that pre-core defective HBV represent viral mutants with an increased capacity to resist exogenous alpha interferon. These findings emphasize that characterization of HBV genome variability prior to interferon therapy may help to predict antiviral response in HBeAg negative patients.

KEY WORDS: viral persistence, genome variability, antivirals

INTRODUCTION

The clinical importance of hepatitis B virus (HBV) genome variability may include some of the mechanisms by which HBV avoids immune clearance and induces chronic infection. Such mutants frequently involve the pre-core region of HBV genome and therefore terminate pre-C/C protein expression [Brown et al., 1992; Carman et al., 1989]. The pre-core/core region of the viral genome specifies the synthesis of a protein, which after proteolytic cleavage is secreted as a 17 kDa molecule commonly referred to as HBe antigen [Garcia et al., 1988; Schlicht et al., 1987]. HBeAg expression, although not required for viral replication or infectivity [Chang et al., 1987; Schlicht et al., 1987], may represent a target at the surface of infected cells for the anti-HBe immune response [Schlicht and Schaller, 1989; Schlicht et al., 1991]. The occurrence of nonsense, frameshift, or initiation codon mutations in the viral pre-core region can arrest the expression of HBeAg without altering the replication capacity of the virus. Such variants have been described around the world in anti-HBe antibody positive chronic HBV carriers and have been shown to arise spontaneously during seroconversion from HBeAg to anti-HBe or during interferon therapy [Brunetto et al., 1991; Günther et al., 1992; Hamasaki et al., 1994; Okamoto et al., 1990]. In HBeAg negative carriers, the most frequently detected mutation is a nucleotide transition at the pre-core codon 28, which converts a UGG into a UAG stop codon [Carman et al., 1989; Li et al., 1990; Okamoto et al., 1990; Tong et al., 1990]. A limiting factor in pre-core mutations is the presence within this region of the pregenome encapsidation signal ϵ, which is essential not only for the packaging of pregenomic RNA but also for the initiation of reverse transcription [Junker-Niepmann et al., 1990; Wang and Seeger 1993; Wang et al., 1994]. Evidence was provided that the pattern of pre-core mutations is restricted by the secondary structure requirements of the ϵ signal, which are essential for its function [Laskus et al., 1994; Lok et al., 1994; Tong et al., 1992, 1993]. Furthermore, it has been demonstrated that HBV genotypes may also influence the rate of occurence of pre-core mutants [Li et al., 1993]. Geno-

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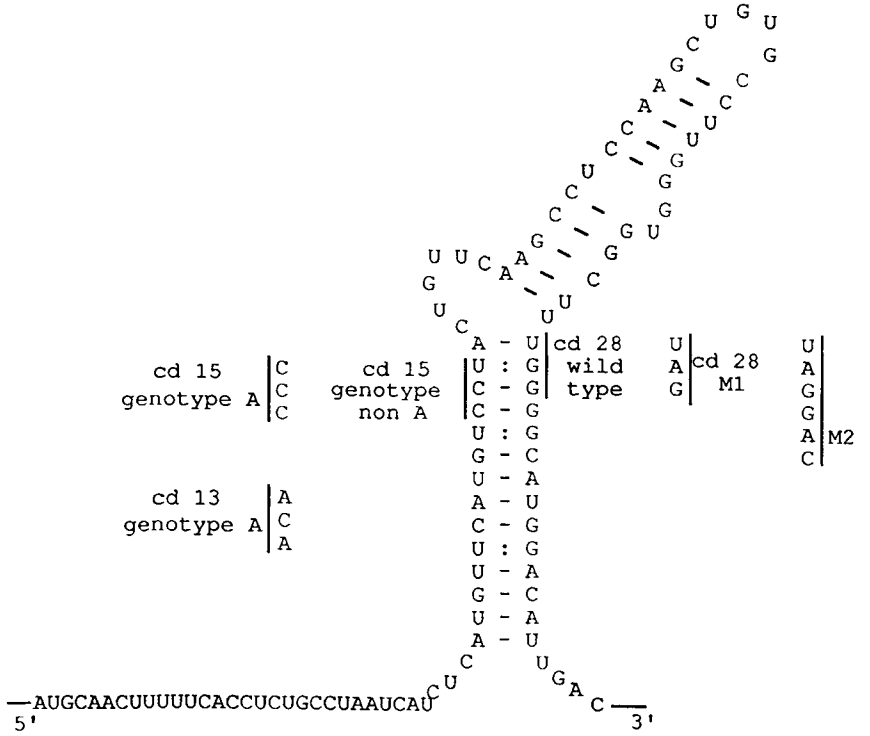

Fig. 1. Proposed secondary structure of the HBV pregenome packaging signal [Junker-Niepmann et al., 1990]. Nucleotide variations between A and non-A genotypes are indicated as well as the two most frequent nonsense mutations (M1 and M2) in the pre-core region.

type A of HBV circulates mainly in Western countries and has a CCC sequence at codon 15 (Fig. 1). The occurrence of a UAG stop codon at codon 28 in HBV genotype A would therefore disrupt a C-G pair present in the wild-type virus, which has been shown to compromise greatly pregenome encapsidation and viral replication. This finding may explain why HBV genotype A rarely circulates as an HBe minus mutant and why genotype D, which is characterized by a CCU sequence at codon 15, is the most frequent viral genotype among the pre-core minus mutants in Western countries (Fig. 1) [Li et al., 1993].

After the emergence of HBe-minus variants, the course of viral infection is characterized by the progression of chronic hepatitis, which may lead to the development of cirrhosis and hepatocellular carcinoma [Fattovich et al., 1988]. Few studies have reported the use of interferon for the therapy of chronic hepatitis B after the emergence of HBe-minus variants [Brunetto et al., 1989, 1993]. However, in this group of patients the influence of the pre-core mutants on the response to therapy and the evolution of these mutants during therapy were not known precisely. In this study, the genetic variability of the pre-core region was analyzed in a group of 35 patients with an HBe minus variant infection during the course of interferon treatment.

MATERIALS AND METHODS

Patients

Thirty-five patients with HBeAg negative chronic hepatitis B were studied (28 males, 7 females; age range 26–72 years; HIV-, HCV-, and HDV-antibody negative; known duration of HBsAg positivity 1–23 years; known duration of anti-HBe positivity 2–13 years). All patients sera were positive for both HBsAg and anti-HBe antibody with raised ALT. Persistent HBV replication was demonstrated in all patients by the detection of serum HBV DNA as assessed by dot blot hybridization (19 patients), branched-DNA assay (28 patients), and polymerase chain reaction (35 patients), and by the detection of viral core proteins in the liver by immunofluorescence (28/30 patients). Liver histology was liver cirrhosis with chronic active hepatitis in 17 patients and chronic active hepatitis in 18 patients. All patients received treatment with recombinant α2b interferon (Schering-Plough, France) at a dose of 1MU (7 patients), 5 MU (4 patients), or 8 MU (24 patients) thrice weekly for a mean duration of 16 months (range 3–40 months). During therapy, patients were monitored monthly for liver function tests and serum markers of HBV replication.

Assays

Serological markers of HBV, HIV, HCV, and HDV were determined by commercial assay kits. HBV DNA was determined semiquantitatively by dot blot hybridization, or quantitatively by branched DNA assay (Chiron, CA) [Zaaijer et al., 1994] and by PCR.

Isolation of DNA

Serum samples (100 μl) were incubated for 4 hours at 37°C in a buffer (25 mmol NaAc pH 6.5, 2.5 mmol EDTA, 0.5% SDS) containing 0.8 mg/ml Proteinase K, followed by phenol-chloroform extraction and precipitation with ethanol. The pellet was resuspended in 50 μl H_2O and used for PCR amplification.

Identification of HBV Genotypes

The method used to identify HBV genotypes was based on size polymorphism in the core gene and the pre-S region as described previously [Li et al., 1993]. Genotype A differs from all other genotypes in having an in-frame insertion of 6 bp near the 3′ end of the core gene, whereas genotypes D and E have an in-frame deletion of 33 bp within the pre-S region. By detecting polymorphism in both regions, HBV genotypes can be divided into three groups: A, B/C, and D/E. Primers C5, C6, and pS4, pS5, whose sequences have been reported elsewhere [Li et al., 1993], were used to detect size polymorphism in the core gene and the pre-S region respectively. Amplification products of the core gene are 105 bp for genotype A and 99 bp for the other genotypes, whereas the size of amplified fragments in the pre-S region is 123 bp for genotypes D/E and 156 bp for genotypes A, B, and C. For samples with small amounts of viral DNA, a distinction between HBV genotypes A (76 bp) and non-A (70 bp) was made possible by a nested PCR in the core gene using a first round of PCR with primers C5 and C6 followed by a second set of amplification using primers C3 and C4 as described previously [Li et al., 1993]. PCR products were analyzed by electrophoresis through 4% Nusieve agarose gels in tris-borate buffer.

PCR Amplification of Pre-Core Region

A one-tenth of extracted DNA was amplified in a 50 μl reaction containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 unit of Taq polymerase (Promega), and 1 pM of each primer (pX: 5′-CTGTAGGCATAAATTGGTCTGCGC-3′ at position 1783–1806, pC: 5′-GTTGACATAACTGACTACTAGGTC-3′ at position 2172–2149). Thirty-five cycles of amplification were undertaken as described previously [Li et al., 1990]. The 390 bp amplification product covered the entire pre-core region and part of the core gene.

Detection of Mutations in the Pre-Core Region

A one-fifth of PCR products was separated by electrophoresis through 2% agarose gel and transferred to nylon membrane (Hybond-N+, Amersham) in 0.4 M NaOH overnight after denaturation in 0.5 NaOH, 1 M NaCl for 30 min. The rapid detection of genetic variability within the pre-core region was carried out by hybridization of amplified products with three oligonucleotides (position 1887–1908) corresponding to the wild-type sequence (M0) and to the two most commonly encountered mutations M1 (one point mutation at position 1896) and M2 (two point mutations at positions 1896 and 1899), as described previously [Li et al., 1990]; see Figure 1. Hybridization with α-^{32}P labelled oligoprobe was performed essentially as described by Li et al. [1990].

Determination of Viral DNA Sequence

The DNA sequence of the pre-core region from selected patients was determined either directly from PCR products or from PCR clones. To obtain single-stranded viral DNA for direct sequencing, 1/25 of the previous conventional PCR product was amplified subsequently asymmetrically (35 cycles) using 1 μM of primer pC and 0.02 μM of primer pX as described by Gunther et al. [1992]. The reaction products were purified by centrifugation through Sephadex G-50 columns and extracted with phenol/chloroform. To obtain PCR clones, amplified products were electrophoresed through a 2% agarose gel in TBE buffer, purified and extracted from the gel using a Geneclean II kit (BIO 101) to remove free primers and dNTPs. The DNA fragment was then cloned into a pGEM-T vector (pGEM-T vector system, Promega). DNA sequencing with primer pX was carried out by the dideoxynucleotide termination method using the sequenase-2 kit (USB) according to the manufacturer's instructions except for minor modifications. Sequencing reactions were run on 6% polyacrylamide/urea gels and autoradiographed with Hyperfilm Beta Max (Amersham).

Competitive PCR for Quantification of Pre-Core Wild-Type and Mutant Genomes

The PCR assay for the quantification of pre-core wild type and mutant genomes was based on the utilization of the same internal standard for both the amplification of the pre-core region and the hybridization with either M0, M1 or M2 oligoprobes. The internal standard was constructed as follows. A 390 bp DNA fragment, containing the entire pre-core region and amplified by PCR using primer pair pX and pC, was obtained from an HBV infected patient and identified by specific hybridization and DNA sequence determination as M2 mutant. The PCR product was purified by Geneclean II kit (BIO 101) and cloned into the pGEM-T vector (Promega). Further digestion with Bgl II (position 1986) and Xba I (position 2143) yielded a modified vector (pGEM-T :M2) with a 157 bp deletion. A 55 bp synthetic DNA fragment containing the sequences of M0 and M1 oligoprobes and that of BgIII and XbaI restriction sites was inserted into the modified vector pGEM-T:M2 to construct an internal standard (pIS), which contains the sequences of wild-type specific oligoprobe (M0) and that of the mutant specific oligoprobes (M1, M2). The pre-core/core region of pIS has a 102 bp deletion as compared to the circulating HBV genomes. DNA sequence determination of pIS confirmed the correct construction of this plasmid.

Competitive PCR for the quantification of the different viral populations was carried out as described by Clementi et al. [1993] with some modifications. Ampli-

fication of template samples in the presence of known amounts of internal standard (pIS) was carried out in a 100 μl reaction containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 pM of each primer (pX, pC), and 1 unit Taq polymerase. Thirty-five cycles of amplification were performed (5 cycles: 94°C 1 min, 48°C 1 min, 72°C 1.5 min; and 30 cycles: 94°C 1 min, 52°C 1 min, 72°C 1.5 min). Each sample was coamplified with four 10-fold dilutions of the pIS within the predicted range of the sample. Fifteen μl of the PCR products were separated by electrophoresis through a 2% agarose gel and then blotted onto nylon membrane. Hybridization was performed with specific probes M0, M1, or M2. The filters were autoradiographed with Hyperfilm MP (Amersham) at −70°C. The relative optical densities of the test sample and that of pIS were determined by a densitometer (NIH image V 1.54 non FPU). Four sample/IS ratios were obtained for each sample (with respect to the four 10-fold dilutions of pIS used for each sample) to determine the quantity of HBV DNA in the sample. The log of sample/pIS band intensities was plotted against the log pIS copy number. The quantity of sample HBV DNA was calculated by the formula of linear regression: $y = a + bx$. When the intensity of the two bands is the same ($y = 0$), the amount of added internal standard represents indirectly the quantity of one viral population (M0 or M1 or M2) present initially in the sample.

RESULTS

Definition of Response Criteria to Interferon Therapy

Patients who responded to antiviral therapy were defined, at the sixth month of therapy, by a decrease of serum ALT levels to the normal range associated with a sustained clearance of serum HBV DNA as detected by branched-DNA assay. Seventeen patients were refered to as responders and two patients eventually cleared HBsAg. Among the 17 responders, a relapse of HBV replication was observed in five patients. Patients without any change in their serum ALT and/or HBV DNA levels and patients with fluctuation of serum ALT and/or HBV DNA levels were considered as nonresponders (18 patients). The initial dose of alpha interferon was not statistically different in responders (mean = 6 MU thrice weekly) and in nonresponders (mean = 5.6 MU thrice weekly) (Mann-Whitney test, $P = 0.96$). The level of HBV replication was assessed by branched-DNA assay prior to therapy and did not differ significantly in responders (mean = 149 10^6 genome eq/ml) and nonresponders (mean = 203 10^6 genome eq/ml) (Mann Whitney test, $P = 0.25$).

HBV Genotype and Pre-Core Mutants in HBeAg Negative Patients Before Interferon Therapy

Results of viral DNA analysis in patients before antiviral therapy are depicted in Table I. Analysis of DNA size polymorphism in the core gene and the pre-S region enabled us to determine the viral genotype that circulated in the studied individuals as shown in Figure 2. In this series of European patients with HBeAg negative chronic hepatitis B, HBV genotype A was found in 10 (28%), whereas genotypes D/E and B/C were found in 21 (60%) and 3 (9%) patients, respectively. One patient (3%) with a very low amount of viral DNA was infected by a non-A viral genotype (see Table I). The genetic variability of the pre-core region at positions 1896 and 1899 was examined by hybridization of the amplified pre-core region with specific oligoprobes M0, M1, or M2 as shown in Figure 2. In 15 patients (43%), viral genomes containing wild-type (M0) and mutated (M1/M2) sequences were detected. A mixed viral population containing M1 and M2 sequences was found in seven patients (20%). In 13 patients (37%) was found a single viral population containing either the M1 sequence (4 patients), the M2 sequence (1 patient) or the wild-type M0 sequence (8 patients) (see Table I).

Identification of Other Pre-Core Mutations in Patients Infected With M0 Reactive Viral Genomes or HBV Genotype A

In our series of HBeAg negative patients, 10 out of the 35 patients were infected with HBV genotype A, six of them in the form of pure wild-type virus. Two additional cases with non-A genotype were also found to circulate as pure wild-type virus (see Table I). To understand how viral infection could persist in these cases of HBeAg negative chronic hepatitis B, viral DNA sequence of the pre-core region was analyzed. This was carried out after direct sequencing of PCR products in 12 patients and sequencing of PCR clones in six. Results are summarized in Table II. Nucleotide changes in the RNA stem-loop structure of the encapsidation signal ϵ were found in seven patients. Two additional nucleotide changes outside the hairpin structure were detected in two other patients. Amino acid changes without termination of pre-core/core expression were found in six patients: a Ser to Phe change at codon 11 in one patient, a Val to Phe change at codon 17 in four patients, a Gly to Glu change at codon 25 in one patient, and a Gly to Asp change at codon 29 in four patients (Table II). In three patients were found mutations abrogating or terminating pre-core/core protein expression: a nucleotide change in the ATG initiation codon (patient no. 6, HBV genotype non-A), a double nucleotide insertion at position 1843 and 1901 resulting in a frameship mutant (patient no. 5, HBV genotype D/E), a nonsense mutation (TGG to TGA) at codon 28 accompanied by the compensatory mutation at codon 15 (CCT to CTC) to preserve base pairing and secondary structure of the encapsidation signal ϵ (patient no. 16, HBV genotype A) (Table II and Fig. 1). In the remaining three patients (no. 4, 14, 33), analysis of the pre-core sequence showed wild-type viral DNA although trace amount of M1 positive virus was detected by PCR.

Correlation of Response Rate to Interferon Therapy With HBV Genome Variability

When viral genotypes were taken into account, patients infected with HBV genotype A had a signifi-

TABLE I. HBV Genotypes and Pre-Core Sequence Heterogeneity in HBe Ag Negative Patients, Before and After Interferon Therapy

	Responders								Non Responders							
			Before IFN			After IFN					Before IFN		After IFN			
	No. patient	Genotypes	M0	M1	M2	M0	M1	M2	No. patient	Genotypes	M0	M1	M2	M0	M1	M2
M0	1	A	+	–	–	*	–	–	18	A	+	–	–	+	–	–
	2	A	+	–	–	–	–	–	19	A	*	–	–	+	–	–
	3	A	+	–	–	–	–	–								
	4	A	+	–	–	+	–	–								
	5	D/E	+	–	–	+	–	–								
	6	nonA	+	–	–	+	–	–								
M1/M2	7	D/E	–	+	–	*	+	–	20	D/E	–	*	+	–	–	+
	8	D/E	–	*	+	–	–	+	21	D/E	–	+	+	–	+	+
	9	D/E	–	*	+	–	–	*	22	D/E	–	+	+	–	–	–
	10	D/E	–	+	+	–	+	+	23	D/E	–	–	+	–	–	+
	11	D/E	–	*	+	–	–	+	24	B/C	–	+	–	–	+	–
									25	D/E	–	+	–	–	–	–
									26	B/C	–	+	–	+	–	–
M0+	12	B/C	+	+	+	*	+	+	27	D/E	+	+	+	+	+	+
M1/M2	13	D/E	+	–	+	–	–	+	28	D/E	*	+	+	–	+	+
	14	A	+	+	–	+	–	+	29	D/E	+	+	+	–	+	+
	15	D/E	+	+	+	–	+	–	30	D/E	+	+	+	+	–	–
	16	A	+	+	–	+	–	–	31	D/E	+	+	+	–	+	+
	17	A	+	–	*	–	–	–	32	D/E	+	+	*	+	+	–
									33	A	+	*	–	+	–	–
									34	D/E	+	+	–	+	+	*
									35	D/E	+	+	–	–	+	–

* = trace amount of viral DNA.

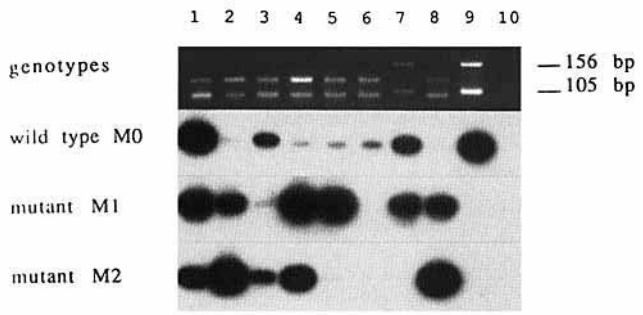

Fig. 2. Determination of HBV genotypes and detection of pre-core mutations by hybridization of PCR products. Viral genotypes were determined by fragment length polymorphism of the core gene and the pre-S region, codon 28 mutants (M0, M1, M2) were detected by specific hybridization as described in Material and Methods. Lane 1 to 9 correspond to patients 27, 28, 30, 29, 32, 5, 16, 10, 3, before interferon therapy. Lane 10 is the result of a negative control.

cantly higher response rate to interferon (7/10 patients) infected with HBV non-A genotypes (10/25 patients) (Xi-2 test, $P = 0.001$) (See Table I). The initial dose of interferon was not statistically different in patients infected with HBV genotype A and in patients infected with non-A viral genotypes (Mann-Whitney test, $P = 0.70$). The initial level of serum HBV DNA tended to be lower in patients infected with genotype A, but this did not reach statistical significance (Mann-Whitney test, $P = 0.10$). It is noteworthy that the two patients who cleared HBsAg (patients no. 3 and 17) were initially infected with HBV genotype A, and the five patients who relapsed were infected with non-A genotypes (patients nos. 6, 7, 10, 11 and 15). When the genetic variability in the pre-core region was examined, it was found that patients infected with a viral population reactive only to the M0 oligoprobe responded to antiviral therapy in 75% of the cases (6/8 patients), whereas patients infected with HBV populations reactive to M1 or M2 oligoprobes did not respond as well to interferon therapy (12/27 patients = 44%) (Xi-2 test, $P = 0.0002$) (see Table I).

Evolution of Different Viral Populations During Interferon Therapy

The evolution of the pre-core mutants was studied during the course of interferon therapy as assessed by hybridization of the amplified pre-core region with specific oligoprobes (M0, M1, M2). In patients who responded to interferon therapy, viral genomes reactive for M0 probe disappeared or significantly decreased in 7/12 patients during therapy, whereas a disappearance or a significant decrease of genomes reactive for M1 or M2 was observed in 5/9 and 3/8 patients respectively (see Table I). In nonresponders, a disappearance of viral genomes reactive for M0, M1, or M2 was observed in 4/11, 5/15, and 3/10 patients, respectively. Overall, M0 reactive HBV genomes were sensitive to interferon therapy in 48% of cases, whereas M1 and M2 reactive genomes were sensitive to interferon in 42% and 33% of cases, respectively (see Table I).

Following this, a competitive PCR assay was set up, as described above, to obtain a quantitative estimation of wild-type and pre-core mutant genomes during anti-

TABLE II. Results of Nucleotide Sequence Analysis of the Pre-Core Region in Patients Infected With Genotype A or M0 Positive Virus

Patient no.	Position and nature of mutations	Direct sequencing	PCR clone	Genotypes	Response to IFN[a]
1	cd 17 GTT-TTT (val-phe) M7 cd 29 GGC-GAC (gly-asp) M9	+	–	A	R
2	cd 17 GTT-TTT (val-phe) M7 cd 29 GGC-GAC (gly-asp) M9	+	–	A	R
3	cd 17 GTT-TTT (val-phe) M7	+	4/4 4/4	A	R
4	wild-type	+	2/2	A	R
14	wild-type	+	–	A	R
17	cd 11 TCA-TTA (ser-leu) M4	+	–	A	R
18	cd 29 GGC-GAC (gly-asp) M9	+	2/5	A	NR
19	cd 17 GTT-TTT (val-phe) M7 cd 25 GGG-GAG (gly-glu) M8 cd 29 GGC-GAC (gly-asp) M9	+	2/3 3/3 2/3 1/3	A	NR
33	wild-type	+	4/4	A	NR
6	cd 1 ATG-ACG (met-thr) M3 cd 13 TCA-ACA (ser-thr) M5 cd 29 GGC-GAC (gly-asp) M9	+	1/6 1/6 1/6	non A	R
16	cd 15 CCC-CTC (pro-leu) M6 cd 28 TGG-TGA (trp-stop) M10	+	–	A	R
5	A insertion in any of nt 23–26 C insertion in any of nt 86–88	+	–	D/E	R

[a]R = responders; NR = nonresponders.

viral therapy. Figure 3 shows the result of a competitive PCR assay with specific hybridization for a serum sample taken from patient no. 27 prior to interferon therapy. The detection limit of the assay was 10 HBV genomes/10 μl serum sample.

The quantity of wild-type and pre-core mutant genomes in serum from seven patients (3 responders and 4 nonresponders) was studied before, during, and after interferon treatment. The results are shown in Table III. In the group of responders, the amount of HBV DNA declined to undetectable levels very rapidly after the beginning of therapy, in parallel to ALT normalization. In patient no. 9, the M2 reactive viral genomes showed a greater resistance to interferon as shown by a slower decrease of this viral population during therapy. In the group of nonresponders, the amount of the different viral genomes decreased without becoming negative in two cases (patients nos. 27 and 34). In patient no. 28, M0 reactive viral genomes disappeared rapidly during therapy, whereas M1 and M2 reactive genomes remained approximately at the same level. In patient no. 20 M1 viral genomes were cleared from serum during therapy, whereas M2 genomes persisted in serum.

DISCUSSION

The evolution of pre-core mutants in HBeAg negative patients suffering from chronic hepatitis B was studied during the course of interferon therapy to clarify further the role of these mutants in viral persistence and pathogenesis of HBV infection. A group of 35 selected patients undergoing antiviral therapy for anti-HBe positive chronic viral hepatitis B was assessed. The overall response rate (17/35 patients, 49%) to interferon therapy was found to be similar to that reported by others [Brunetto et al., 1989, 1993]. Interestingly, the level of HBV replication and the initial dose of interferon in responders and nonresponders were not statistically different. We therefore conducted a study of the evolution of HBV genome variability in these patients during interferon therapy and of viral factors that would help to predict a good response to antiviral therapy.

In this series of French patients, a prevalence of HBV pre-core stop codon mutants of 77% (27 patients) was found and was associated with a M0 positive viral population in 42% of the patients (15 patients). This is in agreement with previous observations in French anti-HBe positive chronic HBsAg carriers [Li et al., 1990, 1993] and with reports from other groups [Brunetto et al., 1991; Hamasaki et al., 1994]. Analysis of HBV genotypes showed a higher prevalence of genotypes non-A (72%), which was expected from the results of previous studies [Li et al., 1993].

A group of 12 patients (35%) who were infected by viral genotype A or did not test positive by PCR for the two most common pre-core mutations (M1 or M2) was identified. This group of patients was of interest since the response rate to interferon therapy was higher (75%) as compared to the rest of the patients. The se-

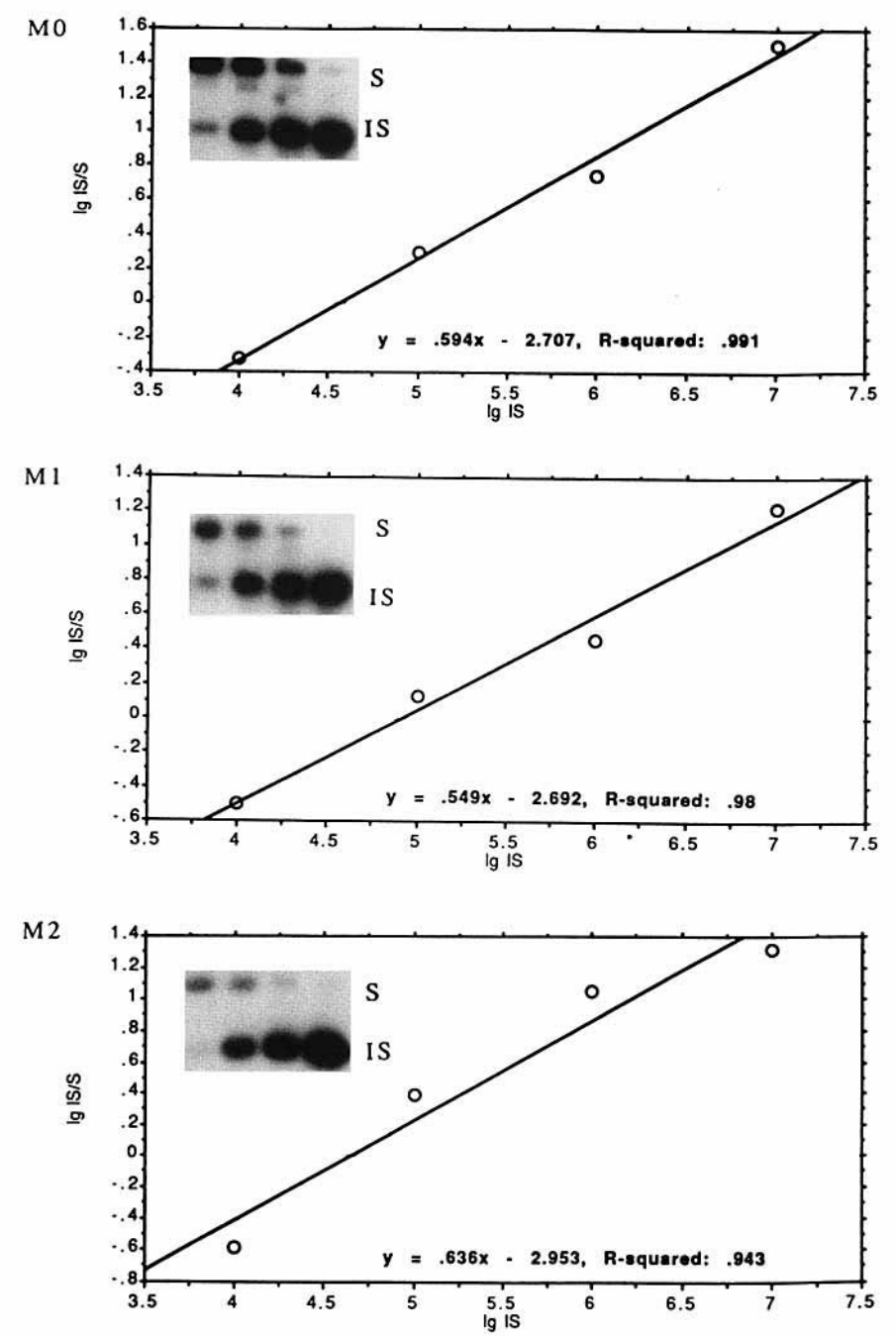

Fig. 3. Quantitative analysis of wild-type and mutated viral DNA in the serum of patient no. 27 after 6 months of interferon therapy. Sample DNA was co-amplified with four 10-fold dilution of internal standard (IS) and hybridized with specific oligoprobes **M0, M1, M2,** respectively. The log of the ratio of band intensities IS/S was plotted against the log of IS copy number. When y = 0 (IS/S = 1), the amount of added IS represents indirectly the concentration of viral DNA initially present in the sample.

quence of the HBV pre-core region was examined in detail and mutations that differed from M1 or M2 and terminated preC/C protein expression were found in three patients. The first one was a mutation of the first initiation codon of the pre-core region and the second was a two-single nucleotide insertion leading to a frameshift, as already described [Lok et al., 1994; Okamoto et al., 1990; Tong et al., 1990]. The third contained a nucleotide change at position 1897 leading to a stop codon, which was associated with a compensatory mutation at codon 15 to restore a correct base pairing in the lower stem of the pregenome encapsidation signal

TABLE III. Evaluation of Treatment Response by a Quantitative PCR Assay for Viral DNA Detection

	No. of patients	Viral populations and ALT[a]	Day 0[b]	Treatment months M2	M6	M12
	1	M0	5.64	0	0	0
		M1	0	0	0	0
		M2	0	0	0	0
		ALT	5 N	1.5 N	N	N
Responders	2	M0	4.01	0	0	0
		M1	0	0	0	0
		M2	0	0	0	0
		ALT	1.7 N	1.1 N	N	N
	9	M0	0	0	0	0
		M1	4.48	0	0	0
		M2	6, 84	3, 75	0	0
		ALT	5 N	1.5 N	N	N
		M0	0		0	0
	20	M1	2.71		0	0
		M2	3.05		0	4.13
		ALT	3.5 N		3 N	N
		M0	7.27		6.12	4.56
	27	M1	7.51		6.57	4.90
		M2	7.43		6.37	4.64
Nonresponders		ALT	2 N		3 N	3.5 N
		M0	1.85		0	
	28	M1	6.23		5.88	
		M2	6.41		4.07	
		ALT	1.5 N		2.5 N	
		M0	4.20		4.78	4.30
	34	M1	4.60		4.96	3.83
		M2	0		0	0
		ALT	4.5 N		12 N	11 N

[a]ALT = serum transaminase (results are given in comparison to the upper limit of normal values).
[b]N = upper limit of normal values.
day 0, or beginning of therapy.

necessary to preserve ϵ function for nucleocapsid assembly and initiation of reverse transcription [Junker-Niepmann et al., 1990; Tong et al., 1992; Wang et al., 1994]. In the remaining nine patients, point mutations in the pre-core region were found and expected to be associated with an amino acid change as already described [Lok et al., 1994]. One mutation at codon 17 (val to Phe), located in the bulge of the hairpin structure of ϵ but outside of the predicted template sequence (UUC) for the priming of reverse transcription [Wang and Seeger 1993], was found in four patients. The influence of this amino acid change on the pre-C/C protein proteolytic processing is now under investigation. These findings underline the great variability of the HBV genome in HBeAg negative patients, and the need to look for new mutations when hybridization with M1 or M2 probes is negative.

Other interesting findings were the higher response rate to interferon in patients infected with HBV genotype A or M0 only positive strains and the greater sensitivity of the wild-type viral population to interferon as compared to strains containing the M2 sequence. This higher response rate to interferon associated with HBV genotype A was independent of both the level of viral replication prior to therapy and interferon doses. These findings suggest that HBeAg negative HBsAg carriers infected with HBV genotype A and/or M0 only positive viral strains represent a distinct subset of cases. This group of patients needs to be examined in more detail to explain why they remain HBeAg negative if M0 positive viral genomes still circulate and why they have a better response rate to interferon. One possibility is that HBeAg production by M0 positive strains is low and overcome by an excess of circulating anti-HBe antibody [Loriot et al., 1995]. Another possibility is the occurrence of mutations in the pre-C/C gene that affect the proteolytic processing of the pre-C/C protein [Garcia et al., 1988], or in the core promoter [Okamoto et al., 1994], which specifically turn down the transcription of the pre-C/C mRNA. The quantitative study of the evolution of the different viral strains during interferon therapy showed that a rapid drop of viral DNA levels occurs at the beginning of therapy in responders. Based on the results of a quantitative PCR assay, the M0 positive viral strains seemed to be more sensitive to interferon as compared to M2 positive strains. The precise understanding of these observations will require the examination of a larger number of

patients receiving different doses of interferon and the replication capacity of HBV genotypes and pre-core mutants in the presence of interferon after transfection of these clones in hepatoma cell lines.

In conclusion, the data show that HBeAg negative chronic HBsAg carriers may respond to interferon therapy although infected with pre-core minus mutants. A subgroup of patients infected with HBV genotype A or viral strains containing nondefective pre-core sequences is more prone to clear HBV DNA from serum and sometimes to eradicate HBV infection. It remains to be determined whether HBV genotype or precore sequence is the primary determinant of response to interferon. These findings emphasize the need for a detailed molecular characterization of viral strains, prior to therapy, to predict the outcome of viral infection following interferon therapy.

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